Molekularna biotehnologija

GS rastline: metode in uporaba

MB3: pogl. 17 in 18

GS rastline: zakaj in kako?

- obstaja več uspešnih tehnik za vnos tuje DNA v rastlinske celice
- na voljo so različni vektorski sistemi za uporabo na rastlinah
- rastlinske celice so večinoma totipotentne (iz 1 transgenske celice lahko vzgojimo celotno rastlino)

- izboljšujemo lastnosti poljščin ali okrasnih rastlin
- GS rastline delujejo kot ceneni ‚živi bioreaktorji' za proizvodnjo proteinov ali metabolitov
- transgenske rastline uporabljamo pri raziskovalnem delu za študij delovanja genov med razvojem rastline in v drugih bioloških procesih

GS rastline: nove lastnosti

Z vnosom enega ali več genov lahko rastlini zagotovimo:

- odpornost proti žuželkam (izražanje insekticida),
- zaščito pred virusi,
- odpornost proti herbicidom,
- zaščito pred patogenimi bakterijami in glivami,
- zakasnitev senescence,
- toleranco pred okoljskimi stresi,
- spremenjeno barvo cvetov,
- povečano prehransko vrednost,
- podaljšano dobo skladiščenja,
- proizvodnjo za človeka zanimivih snovi (zdravila, polimeri, cepiva…)

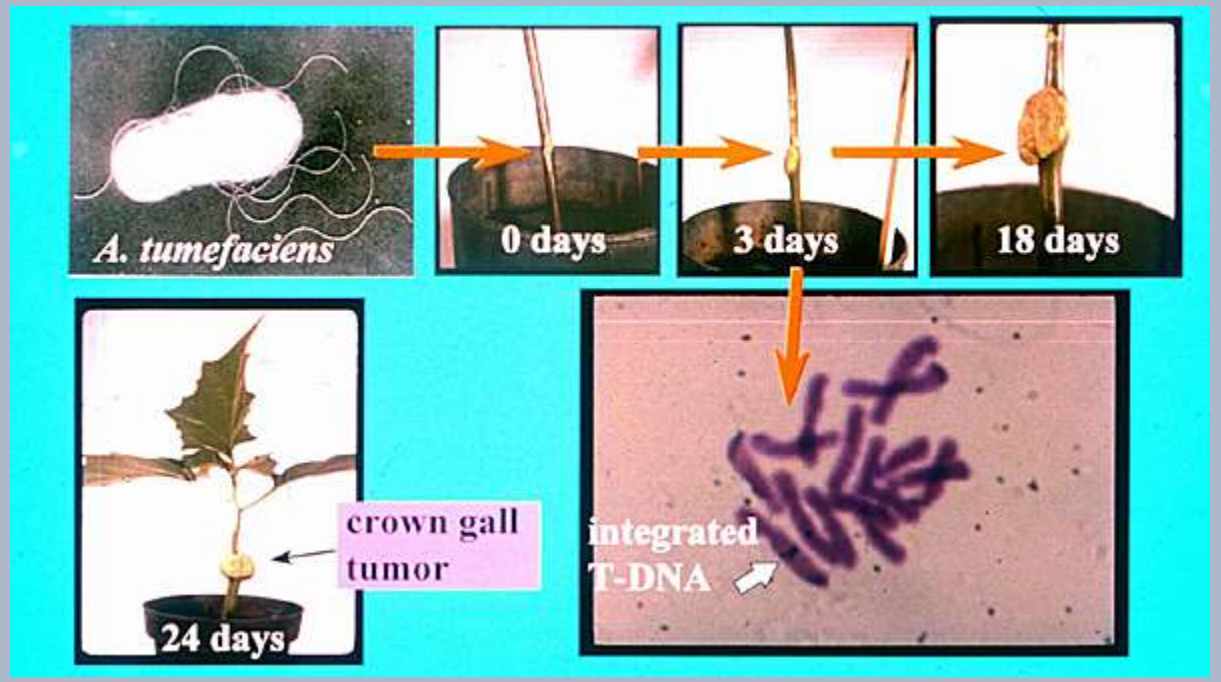
Rastlinska biotehnologija

Gensko spremenjenih je že več kot 140 rastlinskih vrst, pri njihovem razvoju je sodelovalo več kot 50 držav.

Razvoj nove sorte po klasičnih postopkih žlahtnjenja rastlin traja 10 – 15 let, molekularna biotehnologija pa lahko ta čas bistveno skrajša.

http://www.agbios.com/

Crop Name	Events	Phenotypic Trait
Argentine Canola	1	Oxynil herbicide tolerance, including bromoxynil and ioxynil.
Argentine Canola	1	Modified seed fatty acid content, specifically high laurate levels and myristic acid production.
Argentine Canola	2	Glyphosate herbicide tolerance.
Argentine Canola	3	Phosphinothricin (PPT) herbicide tolerance, specifically glufosinate ammonium.
Argentine Canola	1	Imidazolinone herbicide tolerance, specifically imazethapyr.
Argentine Canola	5	Glufosinate ammonium herbicide tolerance and fertility restored.
Argentine Canola	2	Modified seed fatty acid content, specifically high oleic acid, low linolenic acid content.
Carnation	1	Increased shelf-life due to reduced ethylene accumulation through introduction of truncated aminocyclopropane cyclase (ACC) synthase gene; Sulfonylurea herbicide tolerance, specifically triasulfuron and metsulfuron-methyl.
Carnation	2	Modified flower colour; Sulfonylurea herbicide tolerance, specifically triasulfuron and metsulfuron-methyl.
Chicory	1	Glufosinate ammonium herbicide tolerance and fertility restored.
Cotton	2	Resistance to lepidopteran pests including, but not limited to, cotton bollworm, pink bollworm, tobacco budworm.

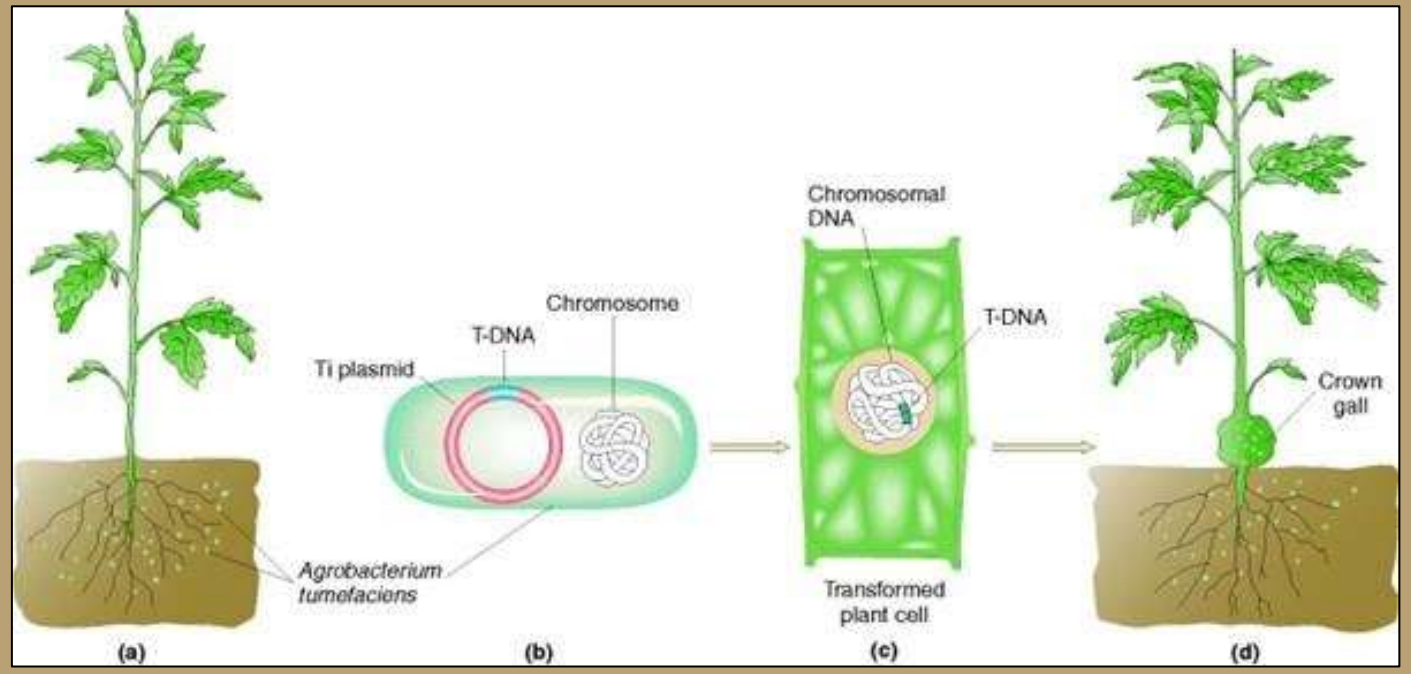


http://www.apsnet.org/edcenter/intropp/lessons/prokaryotes/Pages/CrownGall.aspx

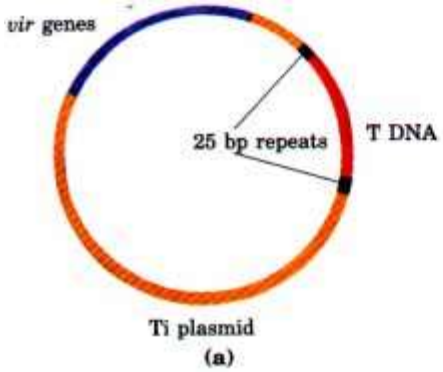
http://www.heritagefruittrees.com.au

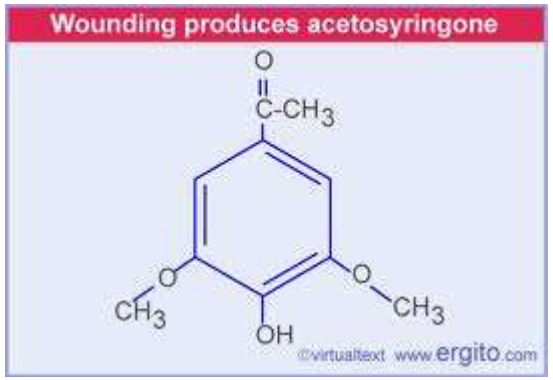
http://www.discoverlife.org

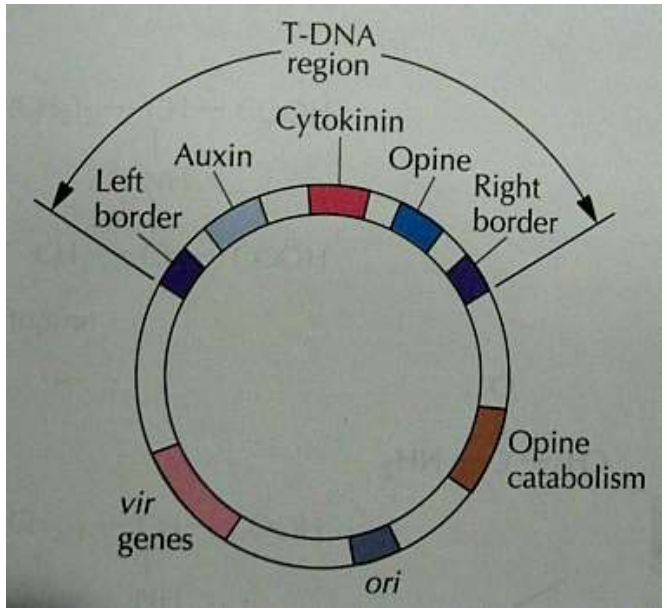
http://www.british-galls.org.uk/gallery.htm

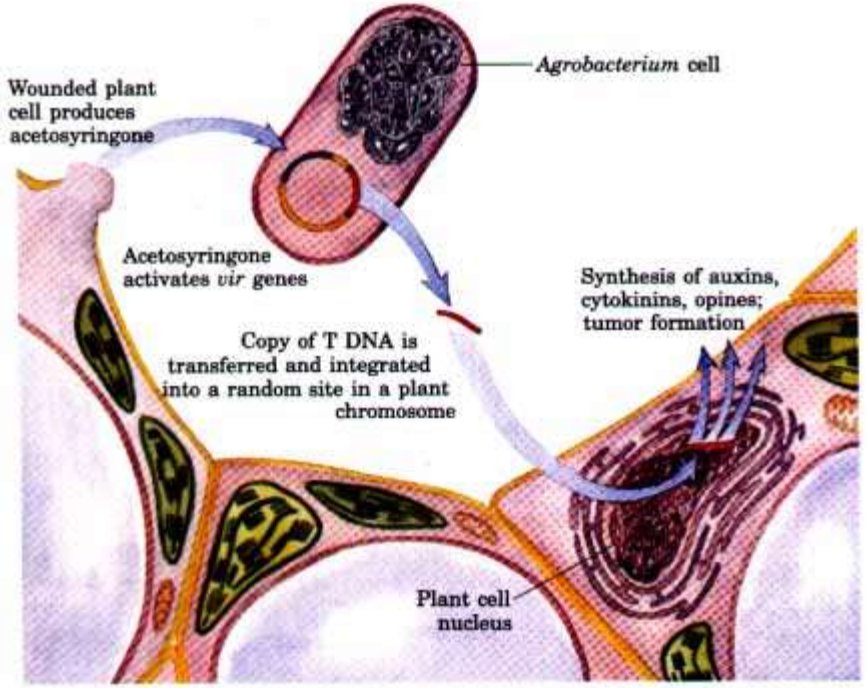


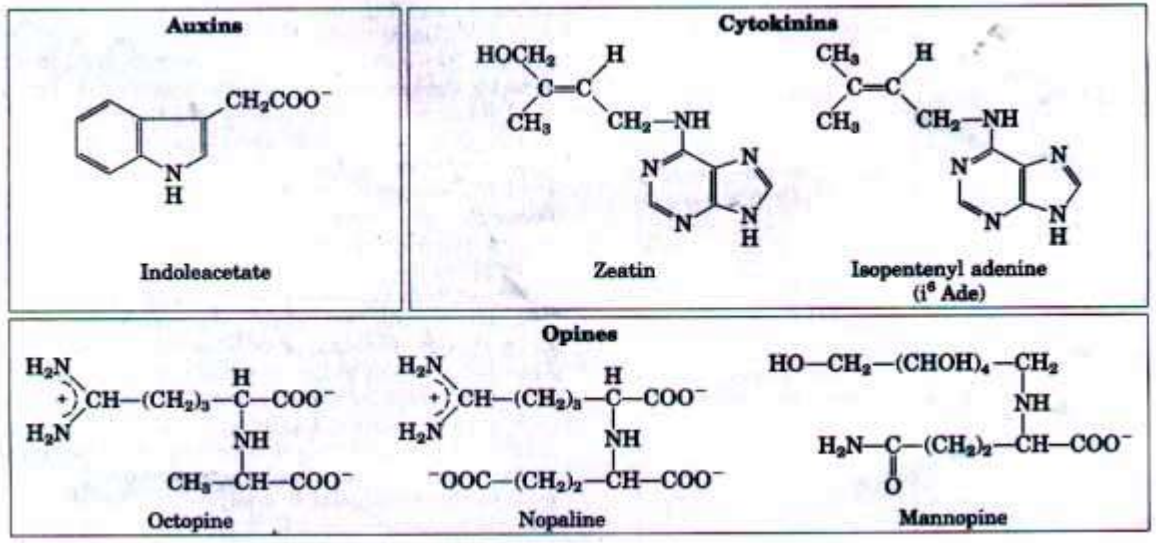
http://www.biologyexams4u.com/2012/12/steps-involved-in-agrobacterium.html


vir genes
25 bp repeats
T DNA
Ti plasmid
(a)


Wounding produces acetosyringone
O
C-CH3
O
CH3
O
CH3
OH
©virtualtext www.ergito.com


T-DNA region
Cytokinin
Auxin
Opine
Left border
Right border
Opine catabolism
vir genes
ori


Agrobacterium cell
Wounded plant cell produces acetosyringone
Acetosyringone activates vir genes
Synthesis of auxins, cytokinins, opines; tumor formation
Copy of T DNA is transferred and integrated into a random site in a plant chromosome
Plant cell nucleus


Auxins
CH2COO−
N
H
Indoleacetate
Cytokinins
HOCH2
H
C=C
CH3
CH2—NH
N
N
N
N
H
Zeatin
CH3
H
C=C
CH3
CH2—NH
N
N
N
N
H
Isopentenyl adenine
(i6 Ade)
Opines
H2N
+
CH—(CH2)3—C—COO−
H2N
H
NH
CH3—CH—COO−
Octopine
H2N
+
CH—(CH2)3—C—COO−
H2N
H
NH
−OOC—(CH2)2—CH—COO−
Nopaline
HO—CH2—(CHOH)4—CH2
NH
H2N—C—(CH2)2—CH—COO−
O
Mannopine

Biosinteza avksina indolocetne kisline (IAA) in citokinina izopentenildifosfata (IPP)

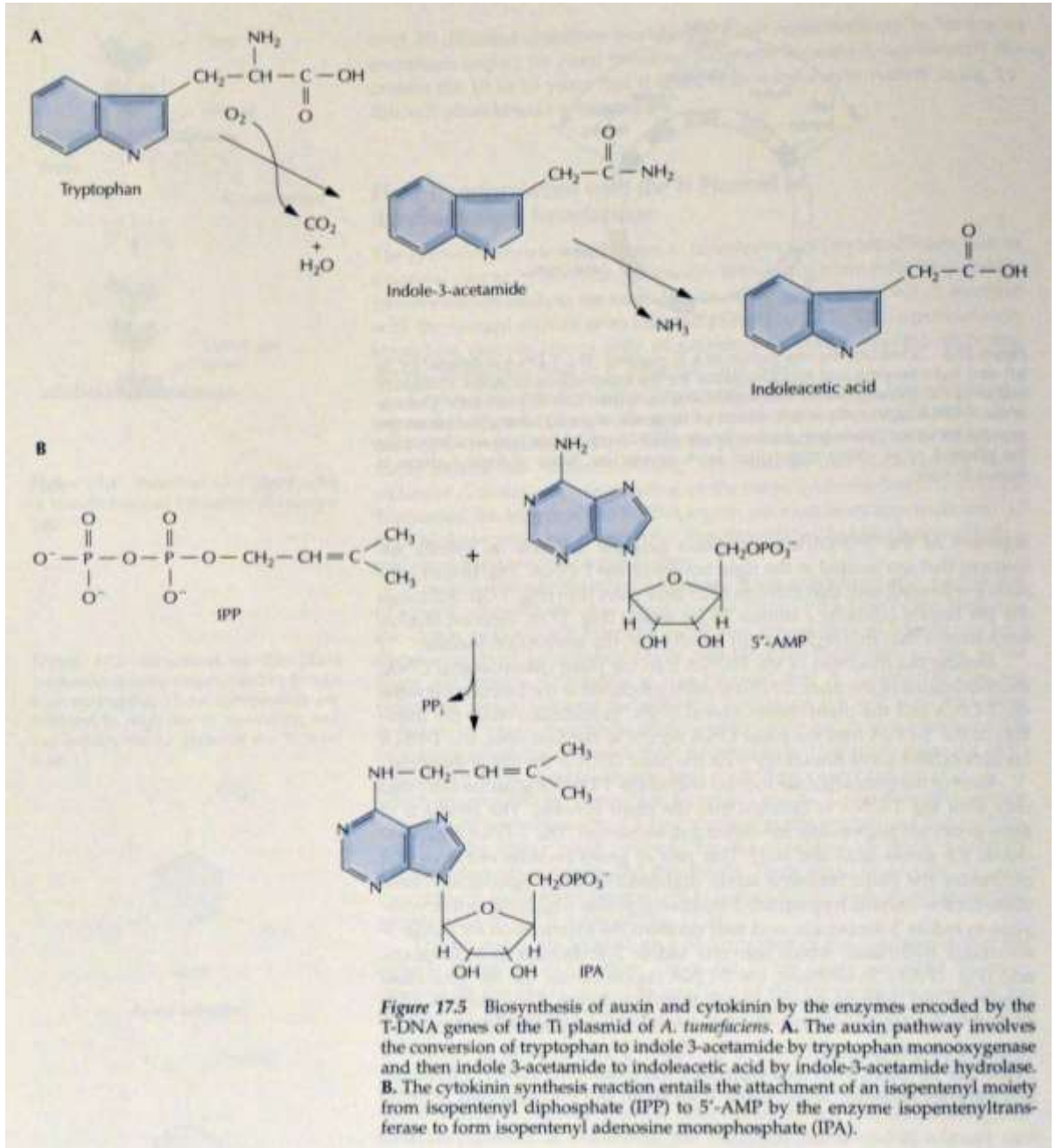
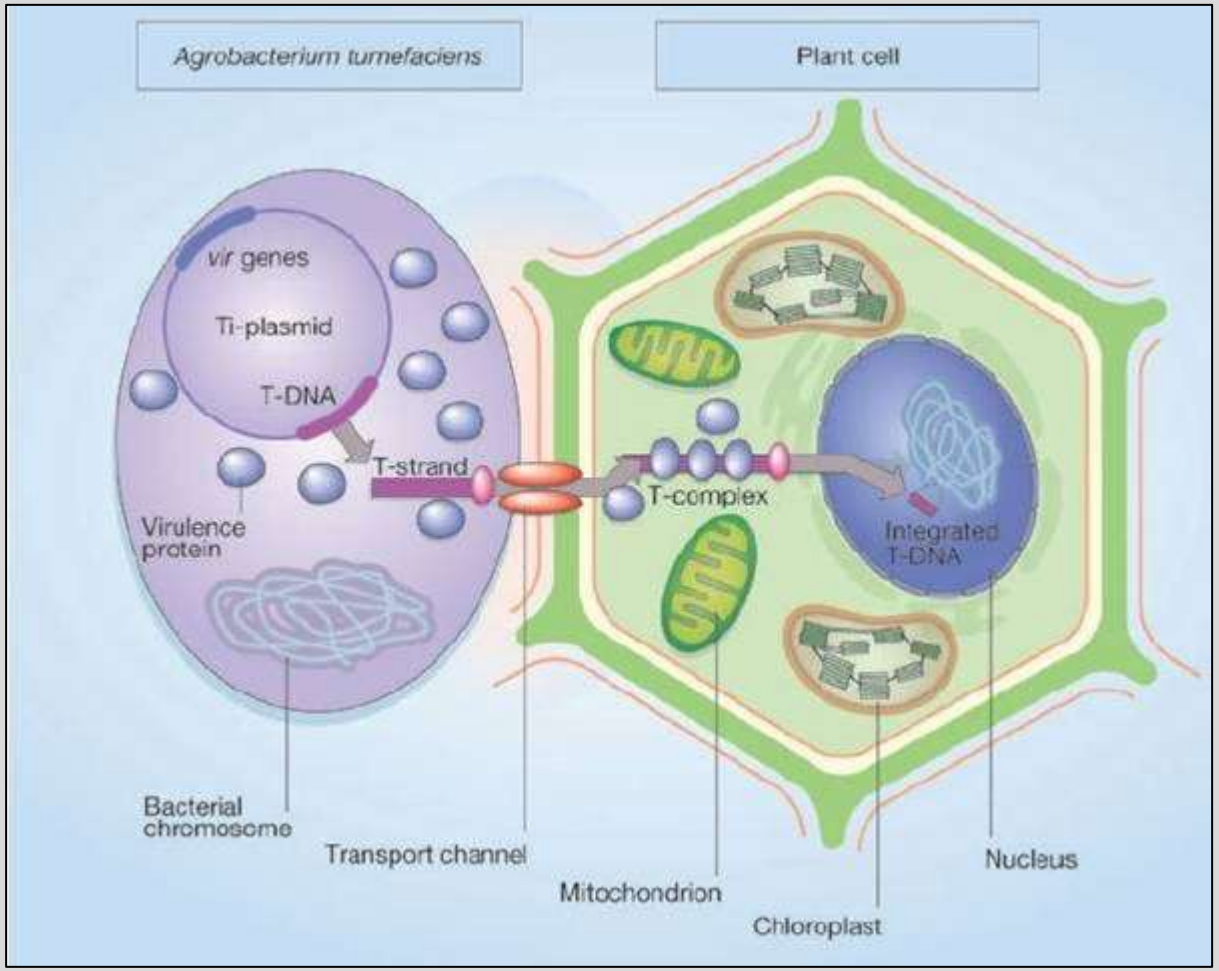


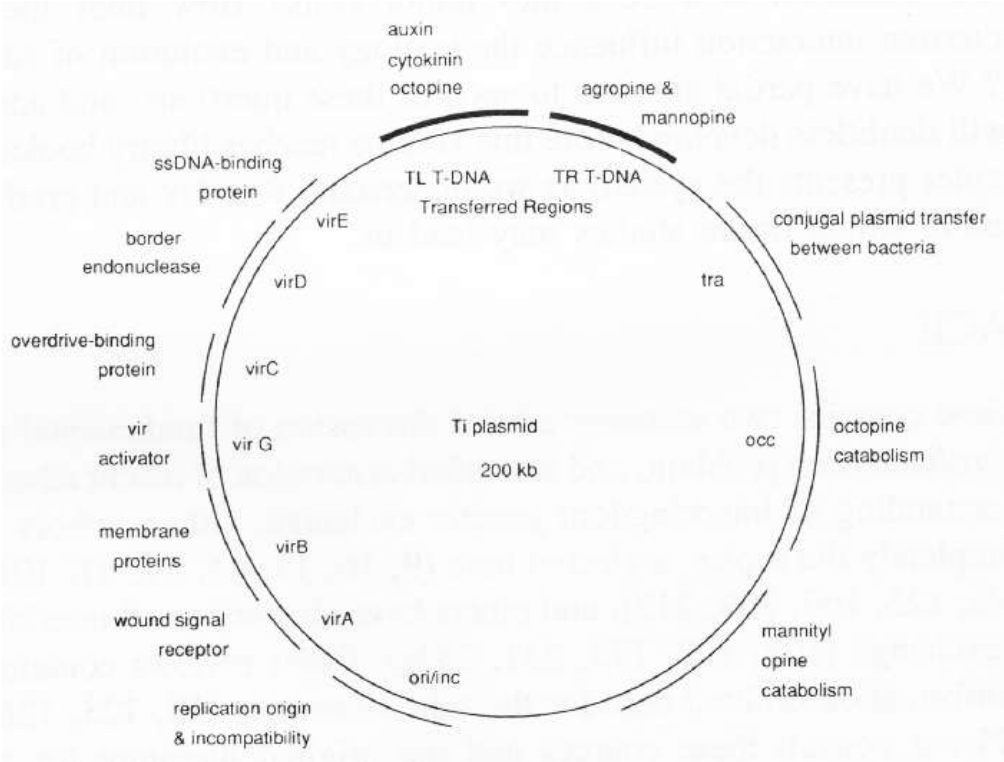
Figure 17.5 Biosynthesis of auxin and cytokinin by the enzymes encoded by the T-DNA genes of the Ti plasmid of *A. tumefaciens*. **A.** The auxin pathway involves the conversion of tryptophan to indole 3-acetamide by tryptophan monooxygenase and then indole 3-acetamide to indoleacetic acid by indole-3-acetamide hydrolase. **B.** The cytokinin synthesis reaction entails the attachment of an isopentenyl moiety from isopentenyl diphosphate (IPP) to 5'-AMP by the enzyme isopentenyltransferase to form isopentenyl adenosine monophosphate (IPA).



http://www.biologyexams4u.com/2012/12/steps-involved-in-agrobacterium.html

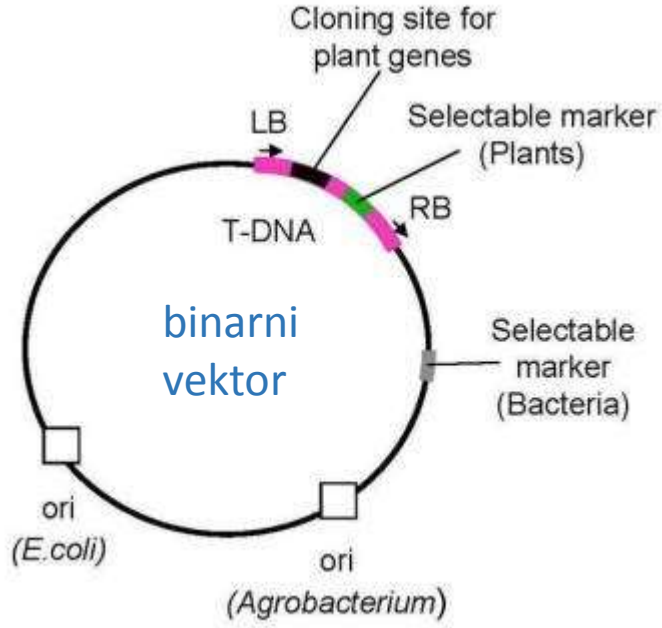
Neprimernost naravnega plazmida Ti za molekularno biotehnologijo:

- velikost (~200 kb) in odstotnost klonirnih mest
- vektor ni kompatibilen z E. coli
- nima genov za selekcijske markerje
- proizvodnja rastlinskih hormonov (regija T) preprečuje regeneracijo nove rastline
- proizvodnja opinov predstavlja neželeno porabo gradnikov



‚Binarni' vektor

Prenosljivi vektor kloniramo v *E. coli*, nato pa ga prenesemo v bakteriji *A. tumefaciens*, ki jo dodatno okužimo z mutiranim ('razoroženim') plazmidom Ti (brez T-DNA, a s funkcionalnim segmentom *vir*).



+

http://www.biologyexams4u.com/2012/12/ti-plasmid-based-vectors-co-integrate.html

Binarni vektor pCB301

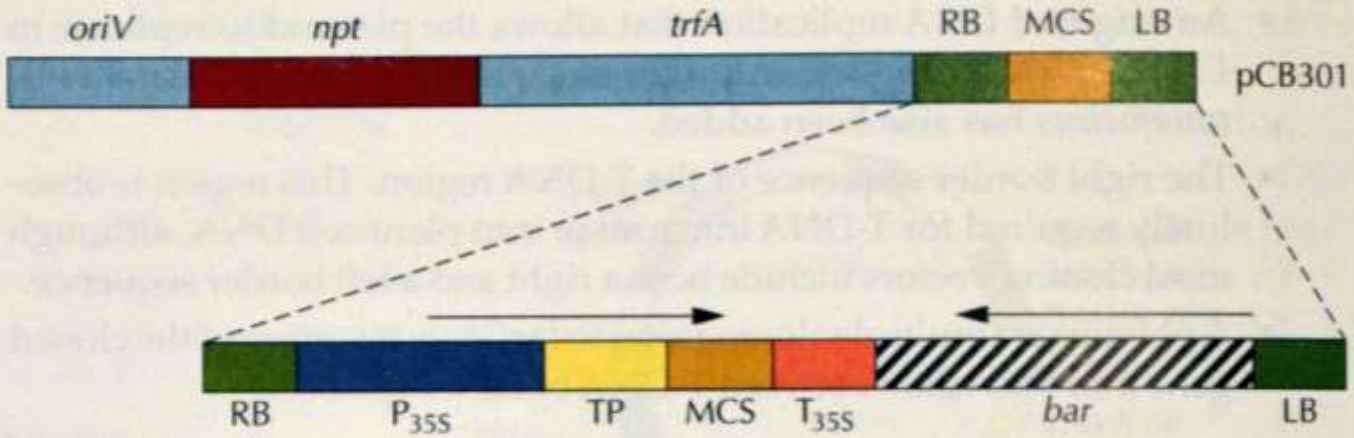
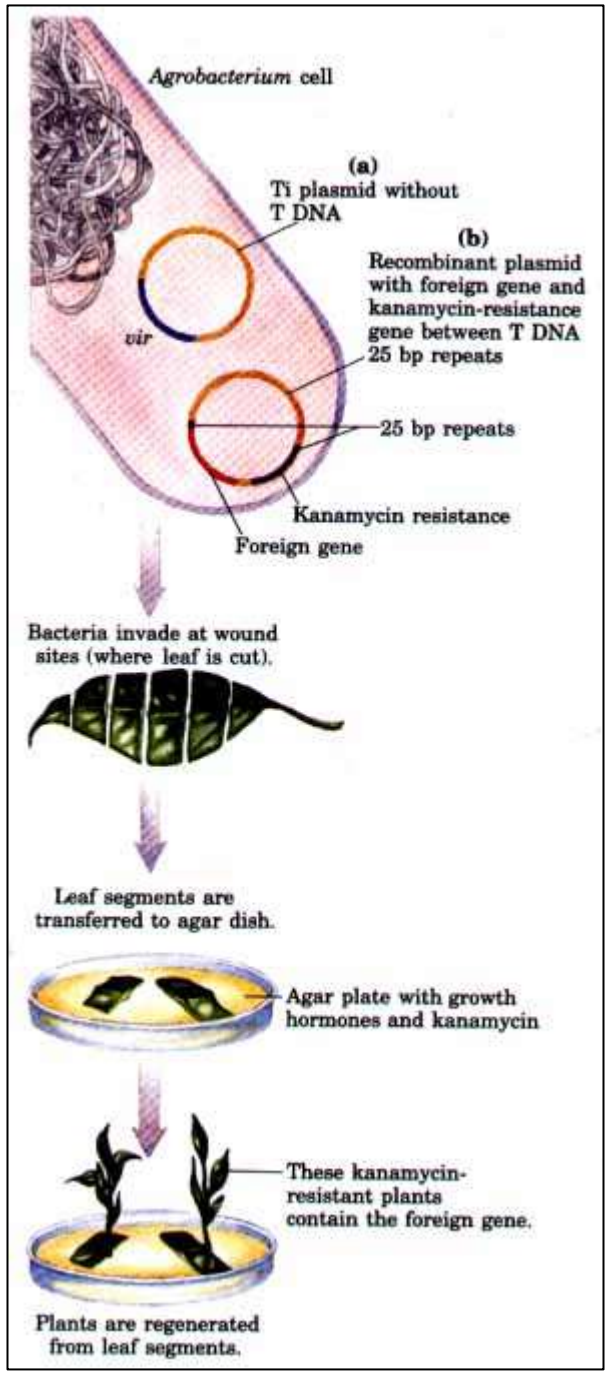
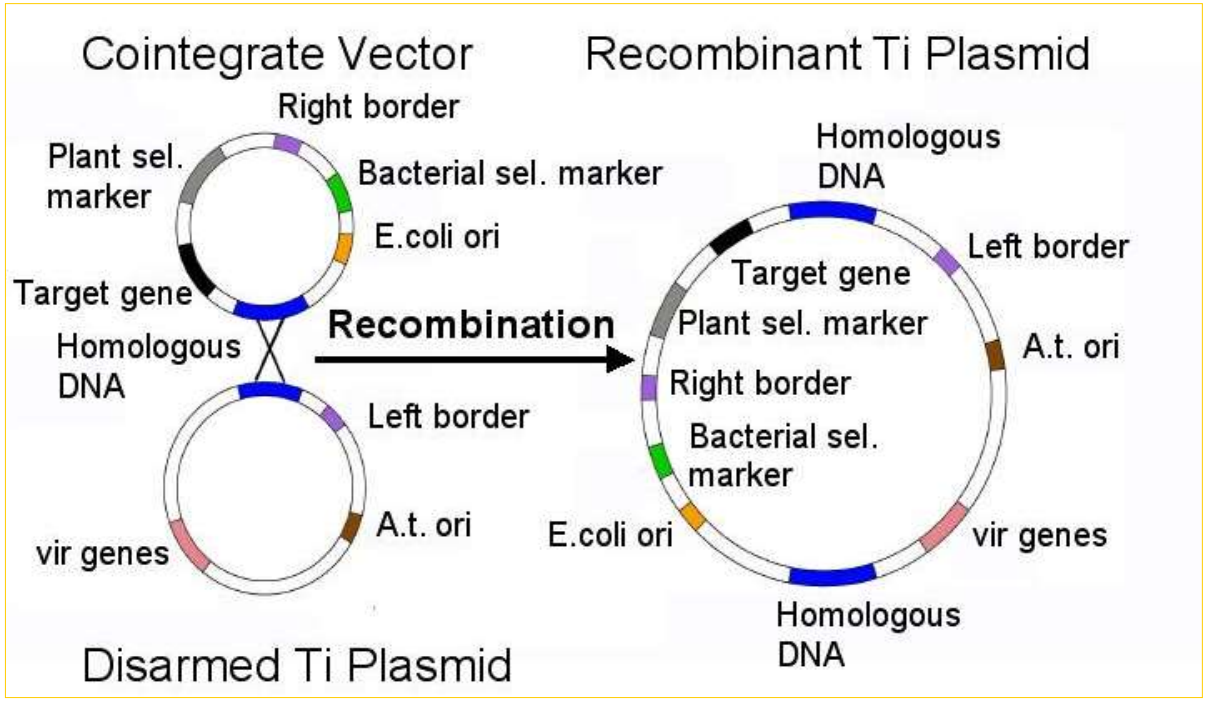


Figure 17.8 The mini-binary vector pCB301 system. Abbreviations: *oriV*, part of origin of replication; *npt*, neomycin phosphotransferase gene; *trfA*, part of origin of replication; RB, right border of T-DNA; MCS, multiple cloning site; LB, left border of T-DNA; P_{35S}, 35S constitutive promoter from cauliflower mosaic virus; TP, targeting protein sequence; T_{35S}, transcription termination sequence from the cauliflower mosaic virus 35S gene; *bar*, gene for phosphinothricin acetyltransferase. By varying the DNA sequence TP, the protein encoded by the introduced gene may be targeted to either the mitochondria or chloroplast. Adapted from Xiang et al., *Plant Mol. Biol.* **40**:711–717, 1999.



‚Kointegrativni' vektor

V *A. tumefaciens* pride do homologne rekombinacije med vektorjem in 'razoroženim' plazmidom Ti, tako da se v tak plazmid Ti vključi celoten klonirni vektor.



Biološki in fizikalni način vnosa

Enokaličnice je mogoče transformirati preko *A. tumefaciens* samo s posebnimi protokoli (npr. embrionalno tkivo potopijo v suspenzijo A.t., potem več dni gojijo brez selekcijskega pritiska in nato precepijo na selektivno gojišče in več tednov gojijo v temi). Odkrili so tudi seve A.t., ki imajo širši spekter tarčnih rastlinskih vrst, ki jo dodatno okužimo z mutiranim (‘razoroženim’) plazmidom Ti (brez T-DNA, a s funkcionalnim segmentom *vir*).

Alternativne metode vnosa temeljijo na transformaciji protoplastov z elektroporacijo ali liposomskimi reagenti, vendar je najučinkovitejša alternativa mikrobombardiranje (biolistika).

http://www.nepadbiosafety.net/subjects/biotechnology/plant-transformation-bombardment

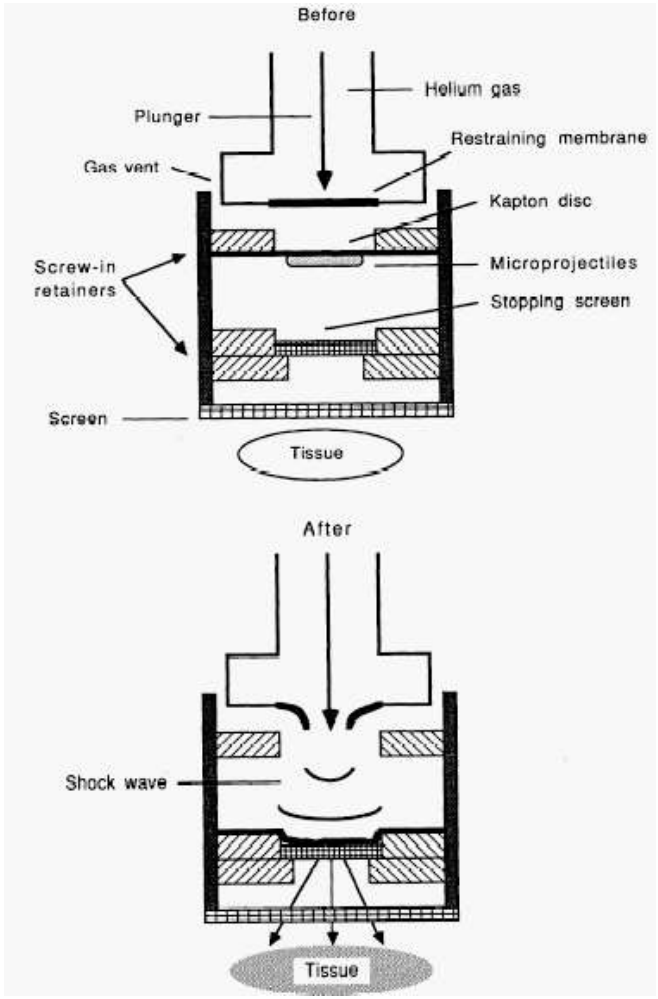
Princip mikrobombardiranja

Delce zlata ali volframa (0,4 – 1,2 um) prekrijemo z oborjeno DNA. Delce pospešimo do 300 – 600 m/s) s stisnjenim helijem. Delci prebijejo celične stene in membrane, v celici se DNA sprosti z delcev in v nekaterih primerih se integrira v genom, včasih v mitohondrijsko ali kloroplastno DNA. V primerih, ko ne pride do vgradnje v genom, lahko zasledimo prehodno izražanje transgena, kasneje pa se vnesena DNA razgradi.

Table 17.3 Transgenic plants formed by microprojectile bombardment of various plant cells

Plant(s)	Cell source(s)
Corn	Embryonic cell suspension, immature zygotic embryos
Rice	Immature zygotic embryos, embryogenic callus
Barley	Cell suspension, immature zygotic embryos
Wheat	Immature zygotic embryos
Turfgrass	Embryogenic callus
Rye	Meristems
Sorghum	Immature zygotic embryos
Pearl millet	Immature zygotic embryos
Orchid	Protocorms
Banana and plantain	Embryonic cell suspension
Poplar	Callus
Norway and white spruce	Somatic embryos
Pea	Zygotic embryos
Cucumber	Embryogenic callus
Sweet potato	Callus
Cranberry	In vitro-derived stem sections
Peony and lily	Pollen
Alfalfa	Embryogenic callus
Bean	Zygotic embryos
Cotton	Zygotic embryos
Grape	Embryonic cell suspension
Peanut	Embryogenic callus
Tobacco	Pollen

Adapted from Southgate et al., *Biotechnol. Adv.* 13:631–651, 1995.



http://www.bio.davidson.edu/courses/molbio/molstudents/

Lastnosti DNA za mikrobombardiranje

Za DNA je bolje, da je linearna, če uporabimo plazmide, pa je bolje, da so manjši kot večji. Kljub temu so uspešno uporabili za vnos z mikrobombardiranjem tudi vektorje YAC (80-550 kb). Uspešnost vnosa so preverili tako, da je YAC na vsakem koncu vseboval po en selekcijski marker, rastline pa so izražale oba. Dolžine nad 150 kb so manj uspešne, vseeno pa ta velikost omogoča vnos celotnih biosinteznih poti.

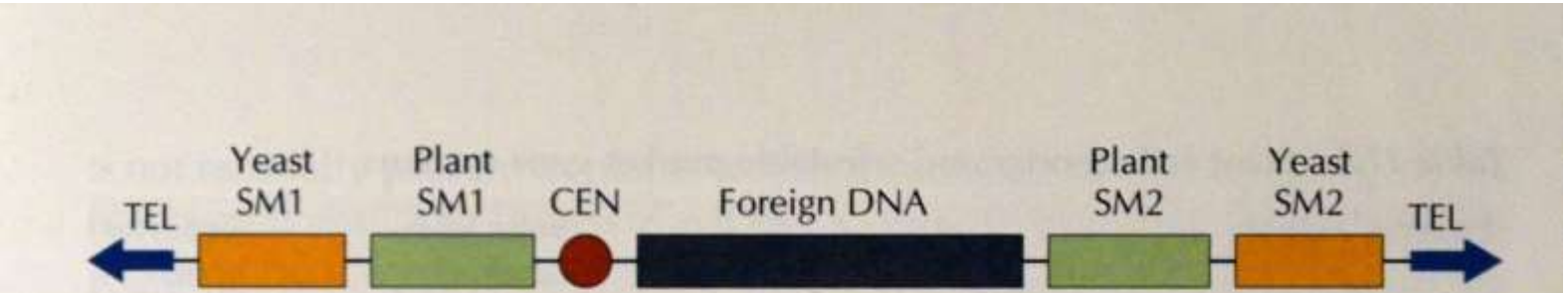


Figure 17.10 Schematic representation of a YAC vector used to transfer large pieces of DNA to plant genomes. Abbreviations: TEL, telomere; SM, selectable marker; CEN, centromere. The various elements are not drawn to scale; the foreign DNA, especially, is much larger than shown. Each of the plant selectable marker genes contains its own promoter and transcription terminator (not shown). The plant selectable markers are a hygromycin resistance gene and a kanamycin resistance gene. Adapted from Mullen et al., *Mol. Breed.* **4**:449–457, 1998.

Reporterji in selekcijski markerji

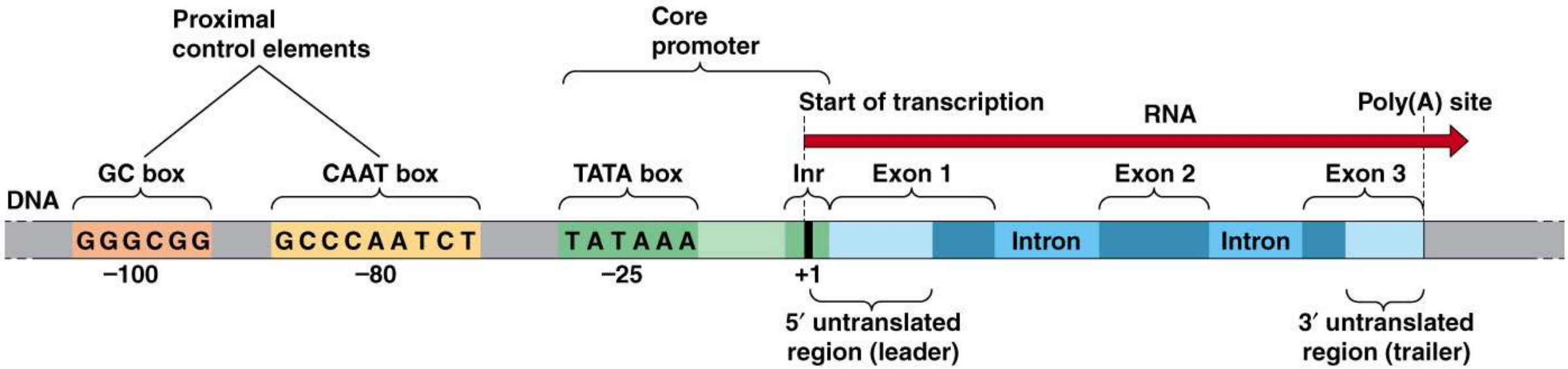
Reporterji omogočajo kvantifikacijo ravni izražanja (transgena), npr. v različnih tkivih transformirane rastline. Nekateri reporterji so hkrati tudi dominantni selekcijski markerji. Ti pogosto izhajajo iz bakterij, a so jih spremenili tako, da imajo regulatorna zaporedja, ki so kompatibilna z rastlinami. Pogosta reporterja sta GUS (beta-D-glukuronidaza) in GFP ter njegovi derivati. Za GUS uporabimo substrat 5-bromo-4-kloro-3 indolil beta-D-glukuronsko kislino ali fluorogene substrate. Za GFP rastline obsevamo z UV ali modro svetlobo. Fluorescenca netransformiranih rastlin je v rdečem delu spektra, transformiranih pa v zelenem.

Table 17.4 Plant cell reporter and selectable marker gene systems

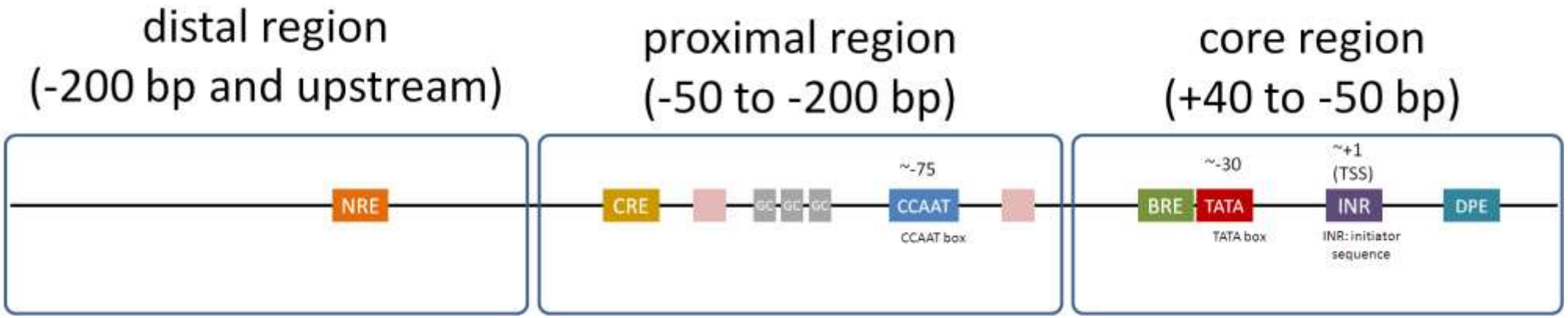
Enzyme activity	Selectable marker	Reporter gene
Neomycin phosphotransferase	Yes	Yes
Hygromycin phosphotransferase	Yes	Yes
Dihydrofolate reductase	Yes	Yes
Chloramphenicol acetyltransferase	Yes	Yes
Gentamicin acetyltransferase	Yes	Yes
Nopaline synthase	No	Yes
Octopine synthase	No	Yes
β-Glucuronidase	No	Yes
Streptomycin phosphotransferase	Yes	Yes
Bleomycin resistance	Yes	No
Firefly luciferase	No	Yes
Bacterial luciferase	No	Yes
Threonine dehydratase	Yes	Yes
Metallothionein II	Yes	Yes
enol-Pyruvylshikimate-3-phosphate synthase	Yes	No
Phosphinothricin acetyltransferase	Yes	Yes
β-Galactosidase	No	Yes
Blasticidin S deaminase	Yes	Yes
Acetolactate synthase	Yes	No
Bromoxynil nitrilase	Yes	No
Green fluorescent protein	No	Yes

Adapted from Walden and Schell, *Eur. J. Biochem.* **192**:563–576, 1990, and Gruber and Crosby, p. 89–119, *in* B. R. Glick and J. E. Thompson (ed.), *Methods in Plant Molecular Biology and Biotechnology*, CRC Press, Boca Raton, Fla.

Struktura evkariontskega promotorja in kodirajoče regije

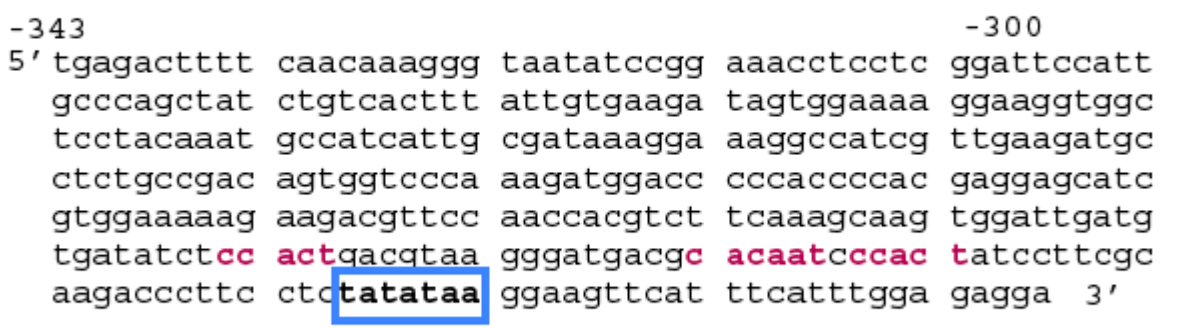


Struktura evkariontskih promotorskih elementov za RNA-polimerazo II



http://genocon.org/assignment-2012/assignment-a/basic-science-a-eukaryotic-promoter/

Nucleotide sequence of the CaMV 35S promoter (-343 to +1)



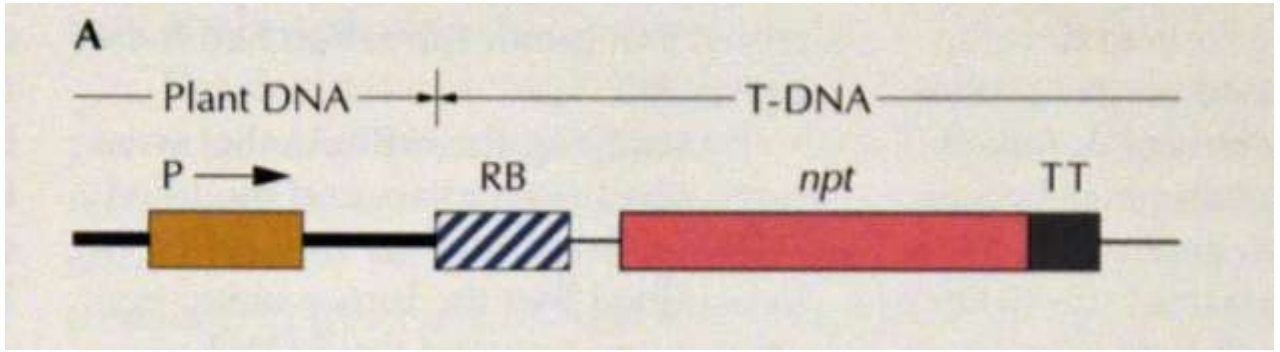
Alternativni promotorji

Promotor CaMV 35S je zelo močen konstitutivni promotor, izražanje pa poteka v vseh delih rastline.

Alternativni promotorji naj bi omogočali:

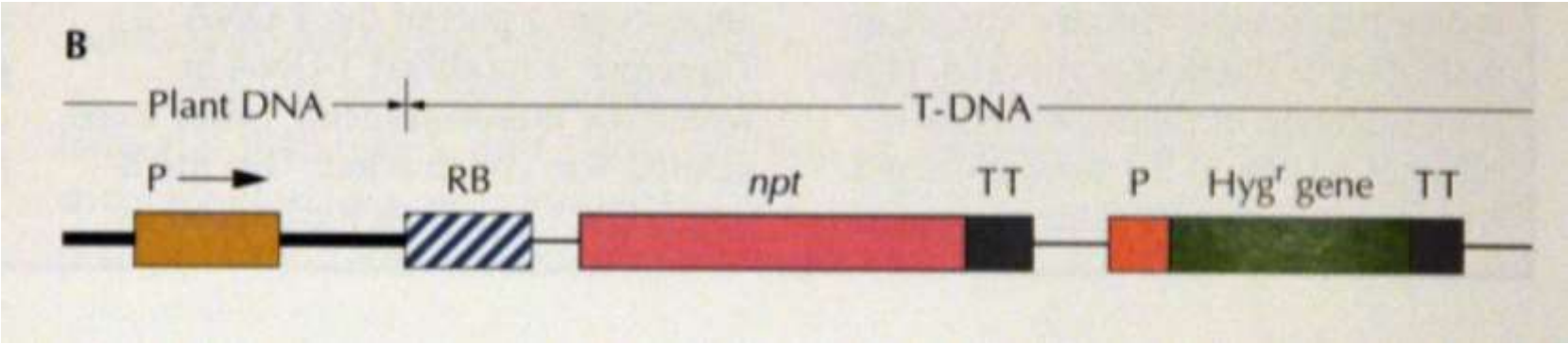
- izražanje v točno določenih tkivih (npr. v zelenih delih rastline – promotor male podenote encima RuBisCo (ribulozabisfosfat karboksilaza))
- izražanje v točno določeni razvojni fazi
- izražanje v točno določenih okoljskih pogojih (npr. s patogenezo povezani promotorji)

Način iskanja promotorjev: s posebnimi vektorji, ki sami ne vsebujejo promotorja, samo z reporterjem v T-regiji v bližini desnega roba. Po prenosu v rastlino, se včasih reporter izrazi – pomeni, da je prišlo do vključitve v neposredni bližini nekega rastlinskega promotorja.



Iskanje promotorjev, ki so aktivni le v določenih razvojnih fazah ali določenih okoljskih pogojih

Kombiniramo reporter brez promotorja in selekcijski marker z lastnim promotorjem (npr. odpornost proti higromicinu). Najprej izberemo celice, ki so odporne proti antibiotiku, preverimo izražanje reporterja z encimskim testom.



Nadaljnje povečevanje ravni izražanja

- ojačevalna zaporedja
- intronske regije
- optimizacija rabe kodona

Eksperimentalna primerjava učinkovitosti promotorskih konstruktov, ki so vsebovali (in/ali):
- promotor 35S
- terminator gena nos
- 1- 7 tandemsko pomnoženih ojačevalnih zaporedij
- ojačevalec translacije (‚omega‘) iz TMV

Najbolj učinkovit je bil konstrukt s 7 kopijami ojačevalnega zaporedja (kar je lahko bilo odvisno od mesta vključitve v genom v konkretnem primeru)

Table 17.5 Testing promoter constructs in transgenic plants

Plant	Average gene expression		Maximum gene expression	
	35S promoter	Composite promoter	35S promoter	Composite promoter
Tobacco	1.0	2.8	2.8	18.3
Rice	1.0	14.4	7.2	47.1

Adapted from Mitsuhara et al., *Plant Cell Physiol.* 37:49–59, 1996.

Usmerjeno spreminjanje genomske DNA pri rastlinah

Spreminjanje genomskih zaporedij s homologno rekombinacijo je pri rastlinah bistveno manj učinkovito kot pri bakterijah ali pri živalih. Zato so razvili drugačno metodo, ki deluje tudi pri živalih. Uporabijo himerno molekulo DNA/(Met)RNA, ki ima večino zaporedja komplementarnega tarčnemu, ne pa celotnega. Ko tako himerno molekulo z mikrobombardiranjem vnesemo v rastlino, pride zaradi nepopolnega ujemanja do sprožitve popravljalnih procesov v celici, takrat pa pride do vgradnje himernega zaporedna v genom. Lahko zamenjamo enega ali nekaj nukleotidov, jih vstavimo ali deletiramo.

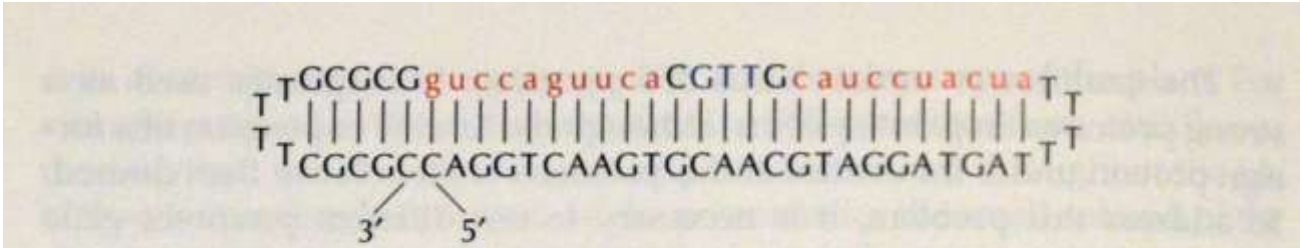


Figure 17.12 An example of a chimeric oligonucleotide used to change the nucleotide sequence of plant DNA. The 2′-*O*-methyl RNA residues are shown in lowercase red letters. The DNA residues are shown in uppercase letters. The codon that was targeted for change is shown in blue. In this case, a CCA codon, which encodes the amino acid proline, that is part of a tobacco acetolactate synthase (ALS) gene was changed to CAA—this is the complementary sequence of the 3′-GTT-5′ sequence shown in blue—which encodes glutamine. Plants with the mutated gene may be selected for by their resistance to the toxic effects of sulfonylurea herbicides, which normally target the ALS enzyme.

Usmerjeno spreminjanje RNA pri rastlinah

Za spremembo fenotipa zadošča sprememba transkriptov, običajno njihova inhibicija.
To lahko dosežemo z uvedbo dodatne kopije gena (kosupresija / RNAi), uvedbo protismernega zaporedja, ribocima ali interferenčne RNA. Npr. koruza s povečanim deležem stearinske kisline in zmanjšanim deležem oleinske kisline je posledica vnosa treh ribocimov, ki razgradijo mRNA za stearoil-ACP delta9-desaturazo. S tem dobijo boljša olja za kuhanje in proizvodnjo margarine.

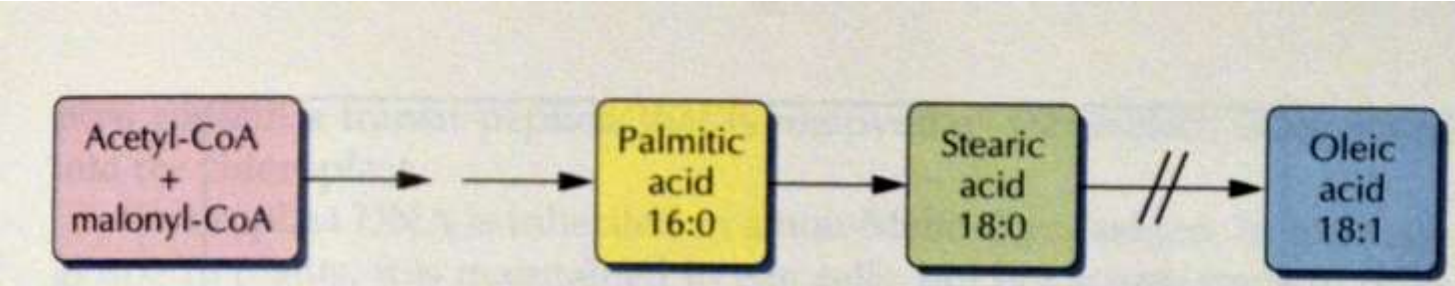


Figure 17.13 Modification of fatty acid biosynthesis in corn. The conversion of stearic to oleic acid is blocked by the action of the added ribozyme on the mRNA encoding stearoyl-ACP Δ9 desaturase, and stearic acid accumulates. The number to the left of the colon designates the number of carbon atoms, and the number to the right of the colon indicates the number of C=C bonds.

Usmerjanje DNA v kloroplaste

Rastlinske celice vsebujejo 1000 – 10.000 kopij kloroplastne DNA (po 50-100 v enem kloroplastu). Tujo DNA lahko vgradimo v kloroplastno DNA na več načinov. Najprej so to izvedli z mikrobombardiranjem s konstrukti, ki omogočajo homologno rekombinacijo. Kasneje so ugotovili, da je pogosto prišlo do vpliva selekcijskega markerja na izražanje sosednjih kloroplastnih genov. Zato so razvili sistem z dvema vektorjema, na enem je gen za selekcijski marker, na drugem pa GOI, vsak z robnimi zaporedji za ciljanje ločenih regij v kloroplastni DNA, ki so jih predhodno določili kot nevtralna mesta. Prisotnost prokariontskih transkripcijskih signalov je omogočalo izražanje samo v kloroplastu, ne pa tudi v jedru. Celice, ki so sprejele gen za selekcijski marker, so v 30 % primerov izražale tudi GOI.

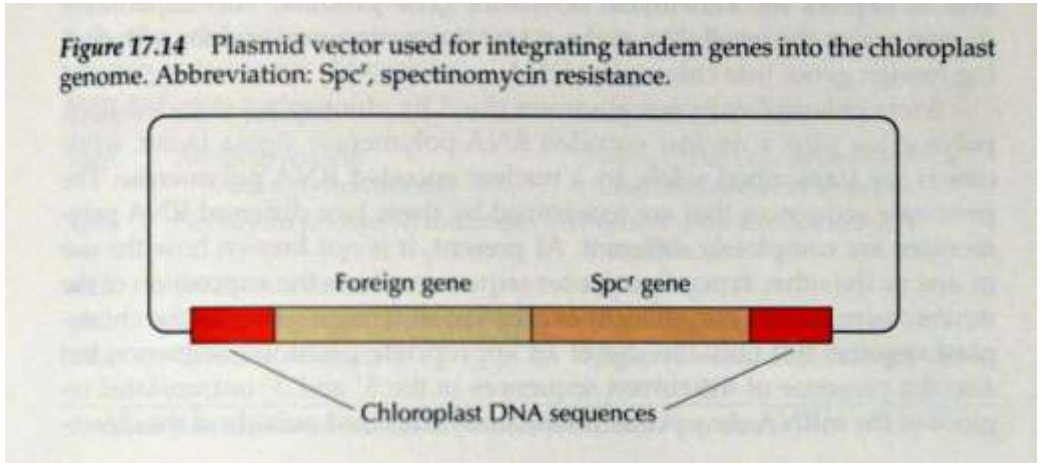


Figure 17.14 Plasmid vector used for integrating tandem genes into the chloroplast genome. Abbreviation: Spc^r, spectinomycin resistance.

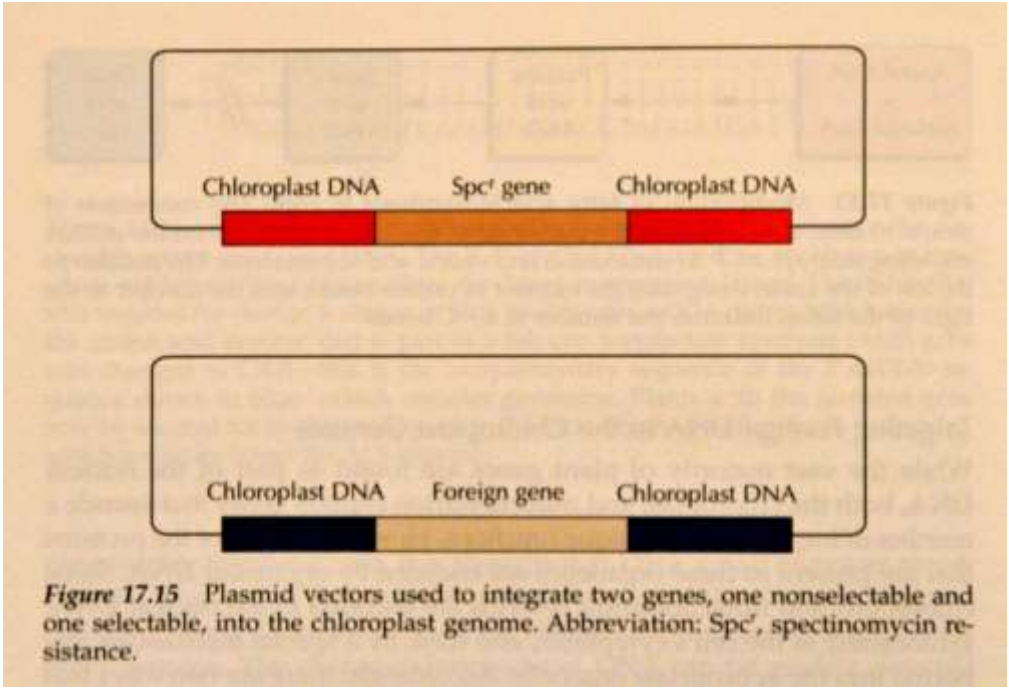


Figure 17.15 Plasmid vectors used to integrate two genes, one nonselectable and one selectable, into the chloroplast genome. Abbreviation: Spc^r, spectinomycin resistance.

Selekcijski markerji in reporterji za kloroplaste

Table 17.6 Some foreign genes that have been used as selectable markers and reporters of transgenic chloroplasts

Gene	Gene product	Function
aadA	Aminoglycoside 3'-adenylyltransferase	Positive selection (spectinomycin and streptomycin resistance)
nptII	Neomycin phosphotransferase	Positive selection (kanamycin resistance)
uidA	β-Glucuronidase	Reporter gene
gfp	Green fluorescent protein	Reporter gene
codA	Cytosine deaminase	Negative selection (5-fluorocytosine sensitivity)

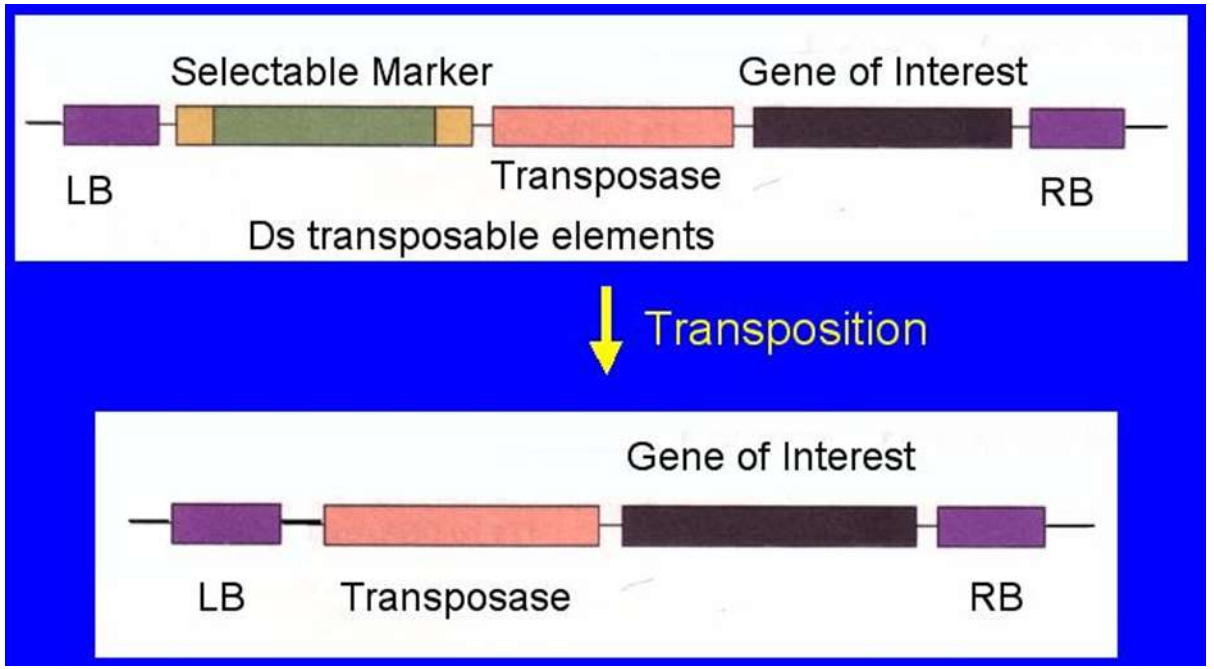
Adapted from Hager and Bock, *Appl. Microbiol. Biotechnol.* **54**:302–310, 2000.

Priprava brezmarkerskih transgenskih rastlin

Čeprav vsebnost selekcijskih in reporterskih genov načeloma za človeka, živali in okolje ni nevarna, pa vseeno ni zaželena. Nekateri so mnenja, da so produkti takih genov lahko toksični ali alergeni, geni, ki omogočajo odpornost proti antibiotikom, pa bi se lahko prenesli na patogene talne mikroorganizme. Razen tega bi vsebnost enega selekcijskega gena onemogočala nadaljnje transformacijske postopke z enakim selekcijskim genom.

Pri postopkih transformacije rastlin z dvema vektorjema, kjer pride do vgradnje na dve različni mesti v genomu, lahko s klasičnimi postopki križanja izselekcionirajo samo tiste rastline, ki so izgubile selekcijski gen.

Drug pristop je z uporabo vektorjev, pri katerih selekcijski gen klonirajo med dva rastlinska transpozicijska elementa (Ds), hkrati pa je v regijo T vključen še gen za transpozazo. Pri vgrajevanju v genom se v ~90 % primerov zgodi, da se selekcijski gen vgradi v genom na nekem oddaljenem mestu glede na GOI, zato ga je mogoče v nadaljevanju odstraniti s konvencionalnim križanjem.



Možne aplikacije rastlinskega genskega inženirstva

- Rastline, odporne proti žuželkam (Bt-toksin, drugi toksini, inhibitorji encimov)
- Rastline, odporne proti virusom (virusni antigeni, RNaza III, rastlinski protivirusni proteini)
- Rastline, odporne proti herbicidom (povečanje števila kopij tarč, zmanjšanje afinitete tarče za herbicid, metabolična inaktivacija herbicida)
- Rastline, odporne proti bakterijam in glivam (salicilna kislina; glukanaze, hitinaze, inhibitorji proteaz, kationski peptidi)
- Rastline, odporne na okoljske strese, npr. suša, povečana slanost, oksidativni stres
- Rastline s podaljšanim rokom uporabnosti, npr. zakasnjeno zorenje in venenje
- Rastline s spremenjeno barvo cvetov
- Rastline s spremenjeno hranilno vrednostjo, npr. več (esencialnih) aminokislin, vitaminov, železa, spremenjena vsebnost lipidov
- Rastline s spremenjenim okusom in izgledom

Primeri izvedbe na naslednjih slikah:

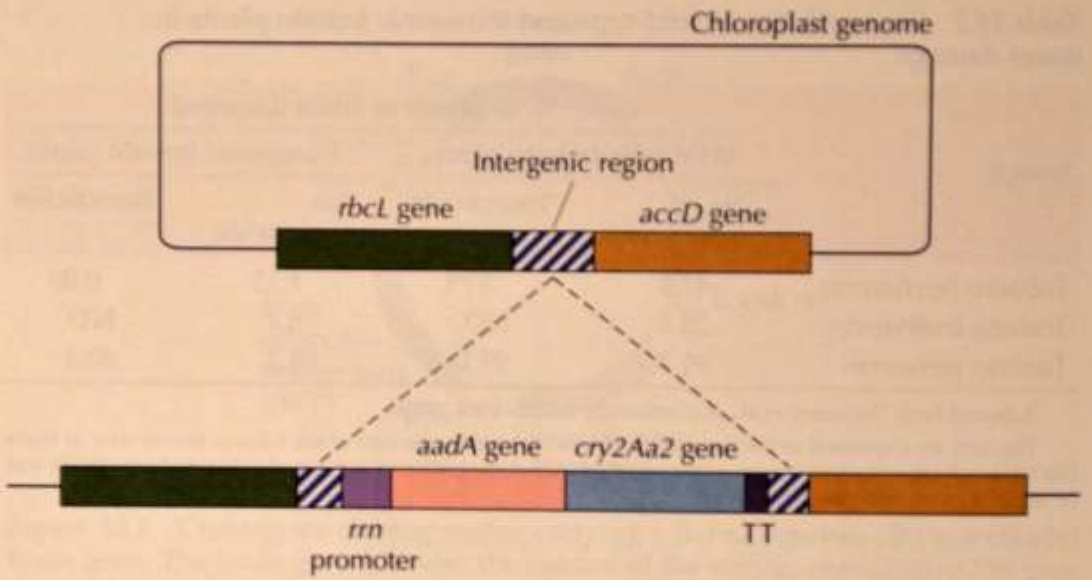


Figure 18.2 Site on the chloroplast genome where a foreign gene encoding the *B. thuringiensis* Cry2Aa2 protoxin is integrated by homologous recombination. The genes *rbcL* and *accD* are both present in a single copy per chloroplast genome. The intergenic region between these two genes, which is the site of insertion of the foreign genes, is smaller than it appears in this representation. The *aadA* gene (encoding spectinomycin and streptomycin resistance) and the *cry2Aa2* gene are both under the transcriptional control of the constitutive chloroplast *rrn* promoter and transcription terminator (TT), and both contain their own ribosomal binding site. Integration of foreign DNA into the intergenic spacer region prevents insertion of a foreign gene from interfering with the expression of any endogenous chloroplast genes. Adapted from Kota et al., *Proc. Natl. Acad. Sci. USA* **96**:1840–1845, 1999.

Table 18.3 Some transgenic plants engineered to have viral coat protein-mediated protection against viral infection

Viral source of coat protein	Transgenic plant
Alfalfa mosaic virus	Alfalfa, tobacco, tomato
Arabis mosaic virus	Tobacco
Beet necrotic yellow vein virus	Sugar beet
Cucumber mosaic virus	Cucumber, tobacco
Cymbidium ringspot virus	Tobacco
Grapevine chrome mosaic virus	Tobacco
Maize dwarf mosaic virus	Sweet corn
Papaya ringspot virus	Papaya, tobacco
Plum pox virus	Tobacco
Potato aucuba mosaic virus	Tobacco
Potato leaf-roll virus	Potato
Potato virus S	Potato
Potato virus X	Potato, tobacco
Potato virus Y	Potato, tobacco
Rice stripe virus	Rice
Soybean mosaic virus	Tobacco
Tobacco etch virus	Tobacco
Tobacco mosaic virus	Tobacco, tomato
Tomato mosaic virus	Tomato
Tomato rattle virus	Tobacco
Tomato streak virus	Tobacco
Tomato spotted wilt virus	Tobacco
Watermelon mosaic virus II	Tobacco
Zucchini yellow mosaic virus	Muskmelon, tobacco

Figure 18.8 Ti plasmid binary cloning vectors containing either the protein-producing sense **(A)** or the antisense RNA-producing **(B)** orientation of the CuMV coat protein cDNA. Each cDNA sequence is under the control of the 35S promoter (P_{35S}) from cauliflower mosaic virus and the transcription terminator–polyadenylation site (*t*RBC) from the gene for the small subunit of ribulose bisphosphate carboxylase. The vector also contains a neomycin phosphotransferase (*NPT*) gene under the control of nopaline synthase transcription signals (*p*NOS and *t*NOS), a spectinomycin resistance (Spc^r) gene, a T-DNA right border sequence, a T-DNA left border sequence, and a broad-host-range origin of DNA replication (*ori*). The protein-producing sense (+) orientation is shown by the A→Z arrow, and the antisense RNA-producing (–) orientation is shown by the Z→A arrow.

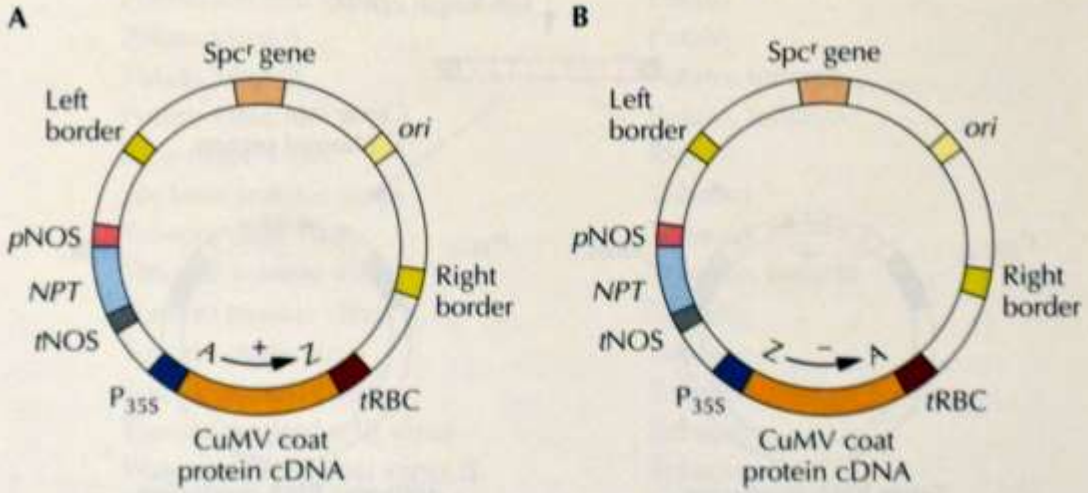
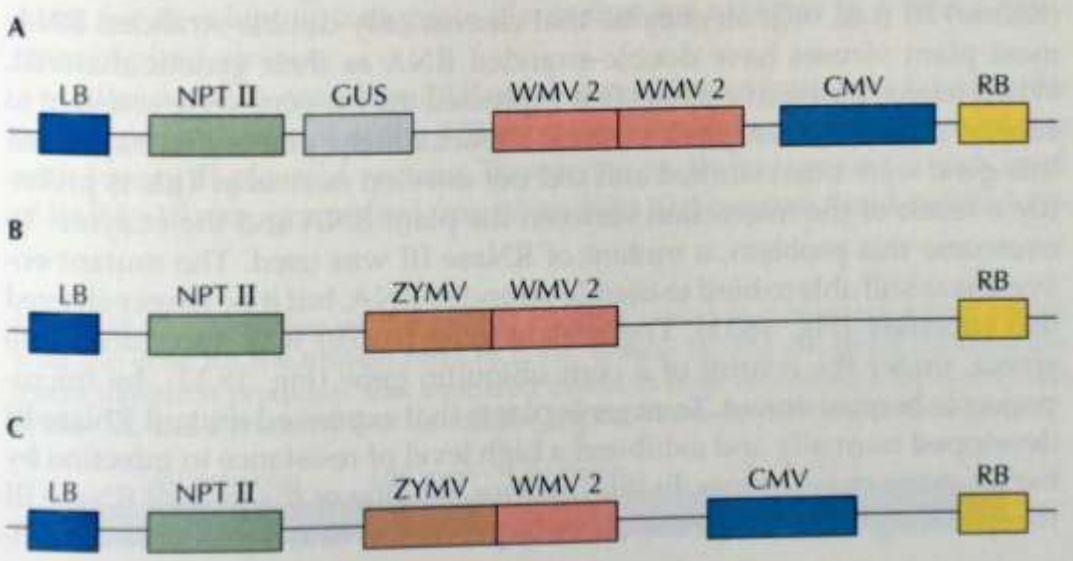


Figure 18.9 **A.** A T-DNA construct with a neomycin phosphotransferase gene (NPT II) as a selectable marker, a β-glucuronidase gene (GUS) as a reporter gene, two copies of the coat protein gene from watermelon mosaic virus 2 (WMV 2), and the coat protein gene from cucumber mosaic virus (CMV). The left and right borders of the T-DNA are indicated by LB and RB, respectively. **B.** Similar to panel A without CMV and GUS, with one copy of WMV 2, and with the coat protein gene from zucchini yellow mosaic virus (ZYMV). **C.** Same as panel B with the addition of CMV. All of the genes in these constructs include both promoters and transcription terminator regions.



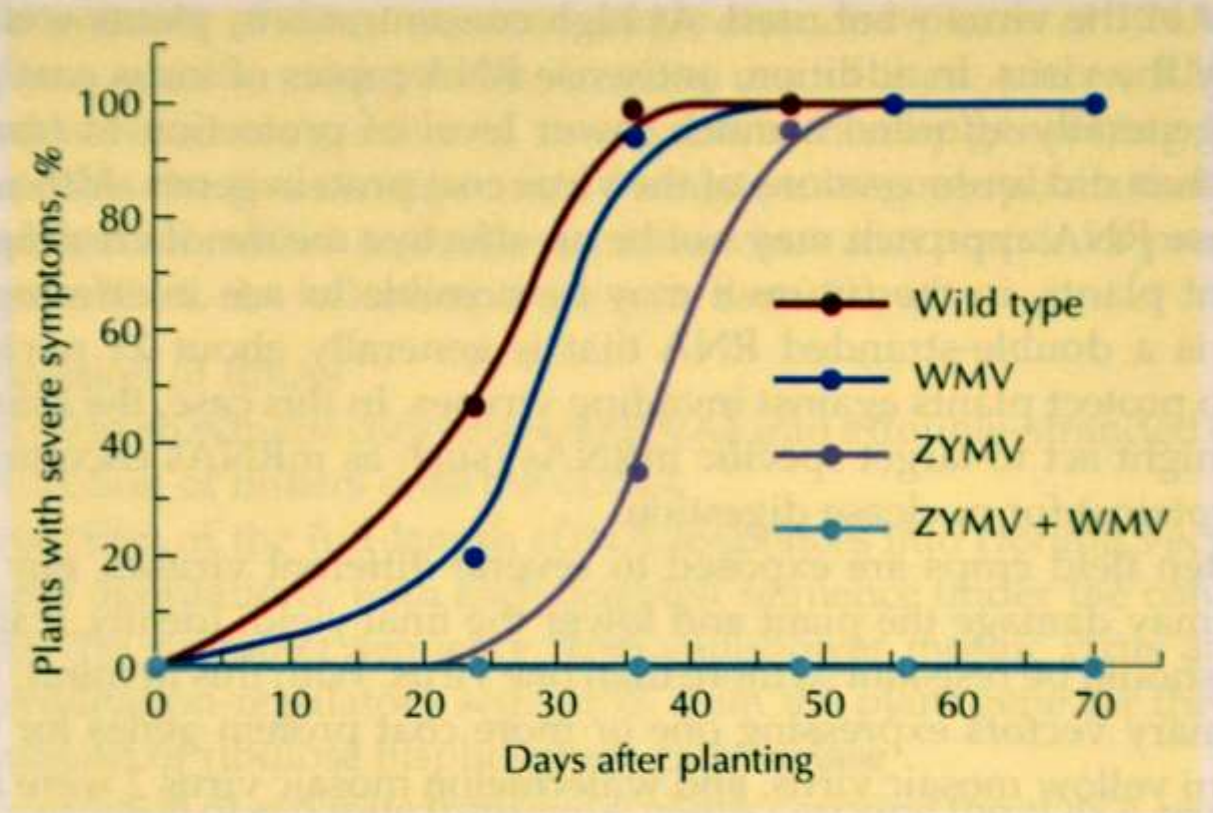


Figure 18.10 Disease frequency in transgenic and nontransformed (wild-type) yellow crookneck squash in the field. Aphids were used to transmit a mixture of zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV) to the squash plants. Adapted from Fuchs and Gonsalves, *Bio/Technology* **13**:1466–1473, 1995.

Table 18.4 Some examples of gene-based herbicide resistance

Herbicide(s)	Mode of development of herbicide resistance
Triazines	Resistance is due to an alteration in the *psbA* gene, which codes for the target of this herbicide, chloroplast protein D-1.
Sulfonylureas	Genes encoding resistant versions of the enzyme acetolactate synthetase have been introduced into poplar, canola, flax, and rice.
Imidazolinones	Strains with resistant versions of the enzyme acetolactate synthetase have been selected in tissue culture.
Aryloxphenoxypropionates, cyclohexanediones	These herbicides inhibit the enzyme acetyl coenzyme A carboxylase. Resistance, selected in tissue culture, is due either to an altered enzyme that is not herbicide sensitive or to the degradation of the herbicide.
Glyphosate	Resistance is from overproduction of EPSPS, the target of this herbicide. Resistance has been engineered by transforming soybean with the gene for a glyphosate-resistant EPSPS and tobacco with a glyphosate oxidoreductase gene, which encodes an enzyme that degrades glyphosate.
Bromoxynil	Resistance to this photosystem II inhibitor has been created by transforming tobacco and cotton plants with a bacterial nitrilase gene, which encodes an enzyme that degrades this herbicide.
Phenoxycarboxylic acids (e.g., 2,4-D and 2,4,5-T)	Resistant cotton and tobacco plants have been created by transformation with the *tfdA* gene from *Alcaligenes*, which encodes a dioxygenase that degrades this herbicide.
Glufosinate (phosphinothricin)	Over 20 different plants have been transformed with either the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *S. viridochromogenes*. The phosphinothricin acetyltransferase that these genes encode detoxifies this herbicide.
Cyanamide	Resistant tobacco plants were produced when a cyananide hydratase gene from the fungus *Myrothecium verrucaria* was introduced. The enzyme encoded by this gene converts cyanamide to urea.
Dalapon	Tobacco plants transformed with a dehalogenase gene from *Pseudomonas putida* can detoxify this herbicide.

Figure 18.14 Overview of systemic acquired resistance in plants. After infection of a plant by a pathogenic fungi or bacterium (shown in blue), the inactive storage compound salicylic acid 2-*O*-β-D-glucoside is converted to salicylic acid, and/or salicylic acid is synthesized. The salicylic acid activates or induces the *NPR1* gene, whose protein product acts as a "master" regulatory protein to turn on the expression of the PR proteins, which have enzyme activity directed against various pathogenic organisms.

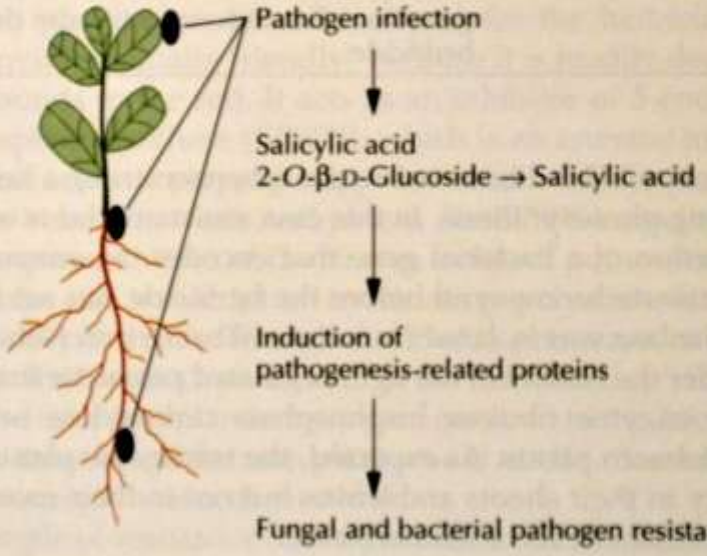


Figure 18.17 The construct used to transform plants so that they constitutively overproduce salicylate. Abbreviations: P_{35S}, the 35S promoter from cauliflower mosaic virus; CTS, chloroplast targeting sequence; ICS, isochorismate synthase; TT, transcription termination region; IPL, isochorismate pyruvate lyase.

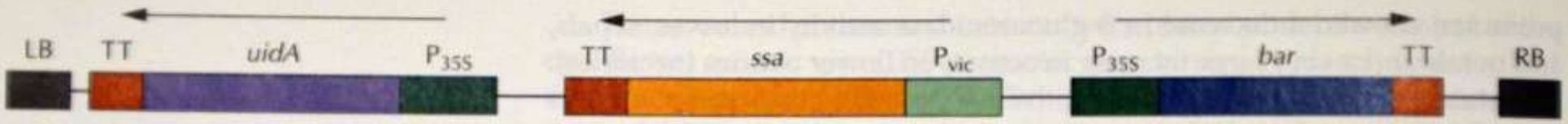


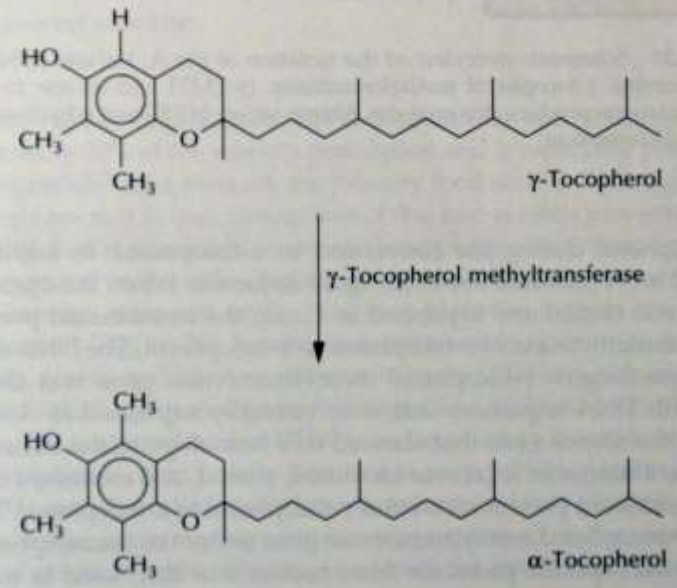
Figure 18.27 Schematic representation of the genetic construct used to transform lupines to increase the methionine content. The arrows indicate the direction of transcription. Abbreviations: LB, left border; P_{35S}, the 35S promoter from cauliflower mosaic virus; *uidA*, a bacterial gene encoding β-glucuronidase; TT, transcription termination region including a polyadenylation site; *ssa*, sunflower seed albumin gene; P_{vic}, the promoter from the pea vicilin gene; *bar*, a bacterial gene that confers resistance to the herbicide phosphinothricin; RB, right border.

Table 18.6 Transgenic canola varieties with modified seed lipid contents

Seed product	Commercial use(s)
40% Stearic	Margarine, cocoa butter
40% Lauric	Detergents
60% Lauric	Detergents
80% Oleic	Food, lubricants, inks
Petroselinic	Polymers, detergents
"Jojoba" wax	Cosmetics, lubricants
40% Myristate	Detergents, soaps, personal care items
90% Erucic	Polymers, cosmetics, inks, pharmaceuticals
Ricinoleic	Lubricants, plasticizers, cosmetics, pharmaceuticals

Adapted from Murphy, *Trends Biotechnol.* **14**:206–213, 1996.

Figure 18.30 The conversion of γ-tocopherol to α-tocopherol by the enzyme γ-tocopherol methyltransferase.



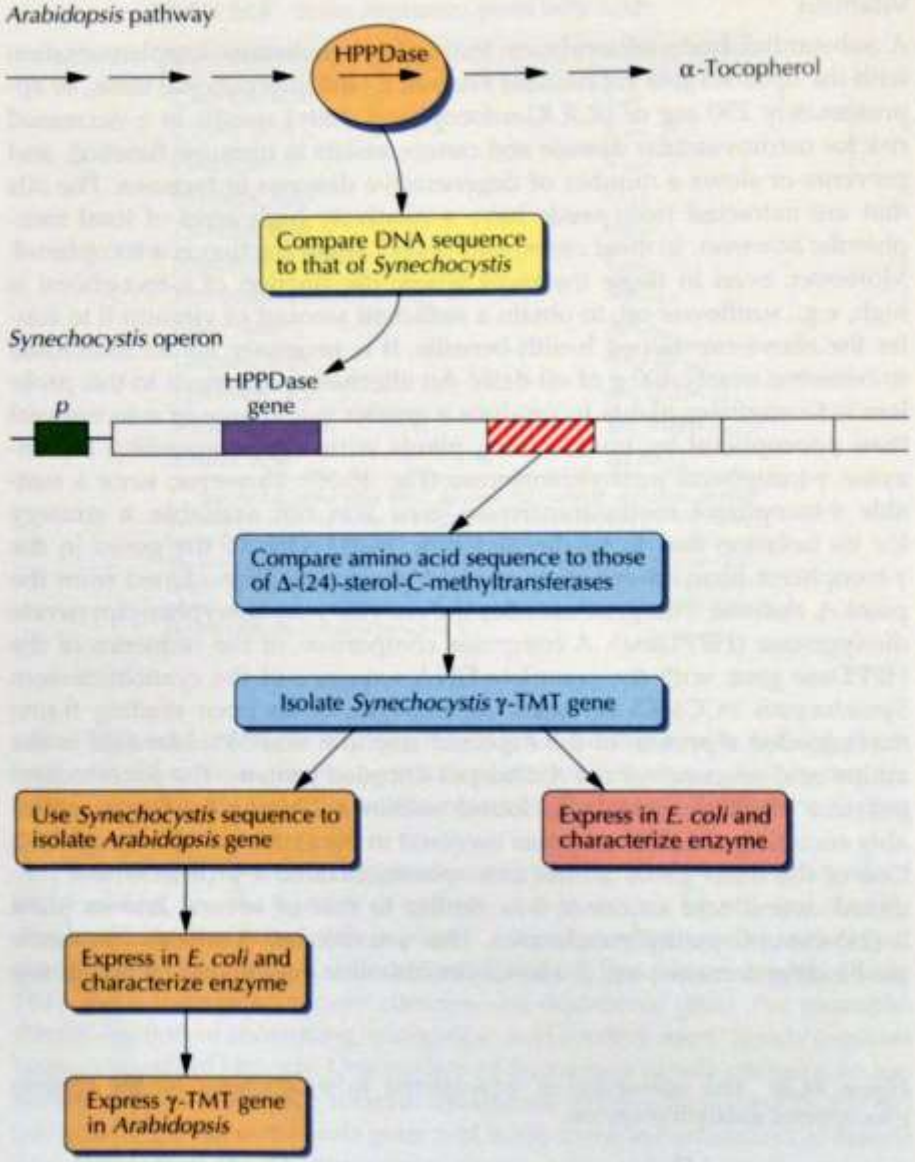


Figure 18.31 Schematic overview of the isolation of the *A. thaliana* cDNA encoding the enzyme γ-tocopherol methyltransferase (γ-TMT) and its use to engineer *A. thaliana* to overproduce this enzyme. Abbreviation: HPPDase, *p*-hydroxyphenylpyruvate dioxygenase.

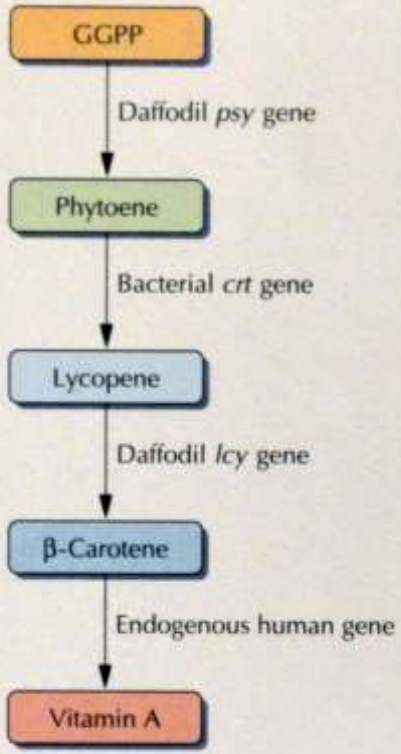


Figure 18.32 The biosynthesis of β-carotene in rice and vitamin A in humans. The phytoene synthase gene (*psy*) was from daffodil and was controlled by a promoter from the rice seed storage protein glutelin. The phytoene desaturase (*crt*) gene was from the bacterium *Erwinia uredovora* and was controlled by the 35S promoter. The lycopene β-cyclase (*lcy*) gene originated from daffodil and was controlled by the rice glutelin promoter. All three genes were fused to transit peptides so that the proteins that they encoded would be transported into the plastid.

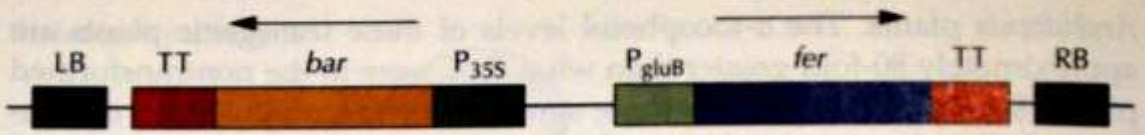


Figure 18.33 The genetic construct used to transform rice and produce ferritin in the seeds. The arrows indicate the direction of transcription. Abbreviations: LB, left border; TT, transcription termination region; *bar*, the bacterial phosphinothricin acetyltransferase gene; P_{35S}, 35S promoter from cauliflower mosaic virus; P_{gluB}, the promoter from the rice seed-storage protein glutelin; *fer*, the soybean ferritin-encoding cDNA; RB, right border.

Table 18.7 Properties of some sweet-tasting proteins

Protein	Plant source	Sweetness factor (weight basis)	Molecular mass (kDa)	Amino acids	Active form
Thaumatin	*Thaumatococcus danielli* Benth	3,000	22.2	207	Monomer
Monellin	*Dioscoreophyllum cumminsii* Diels	3,000	10.7	45 (A chain), 50 (B chain)	AB dimer
Mabinlin	*Capparis masakai* Levl	100	12.4	33 (A chain), 72 (B chain)	AB dimer
Pentadin	*Pentadiplandra brazzeana* Baillon	500	12.0	ND	ND
Brazzein	*Pentadiplandra brazzeana* Baillon	2,000	6.5	54	Monomer
Curculin	*Curculingo latifolia*	550	24.9	114	A_2 dimer
Miraculin	*Richadella dulcifica*	ND	98.4	191	A_4 tetramer

Abbreviation: ND, not determined.

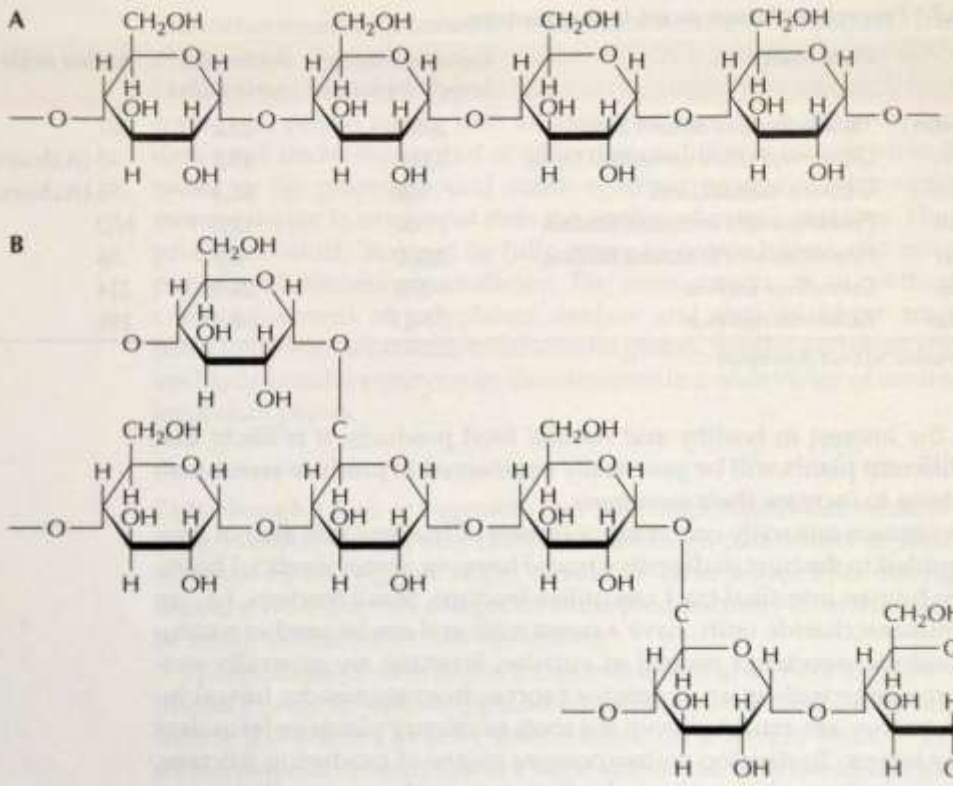
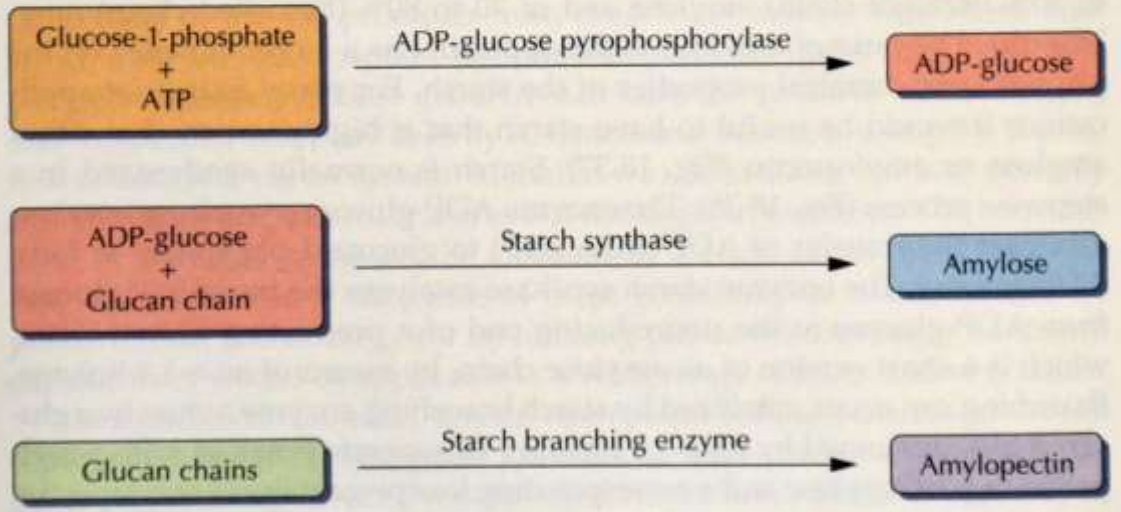


Figure 18.37 A portion of an amylose chain with only α-1,4 linkages **(A)** and a portion of an amylopectin chain with α-1,4 and α-1,6 linkages **(B)**.

Figure 18.38 The major reactions in the biosynthesis of starch.



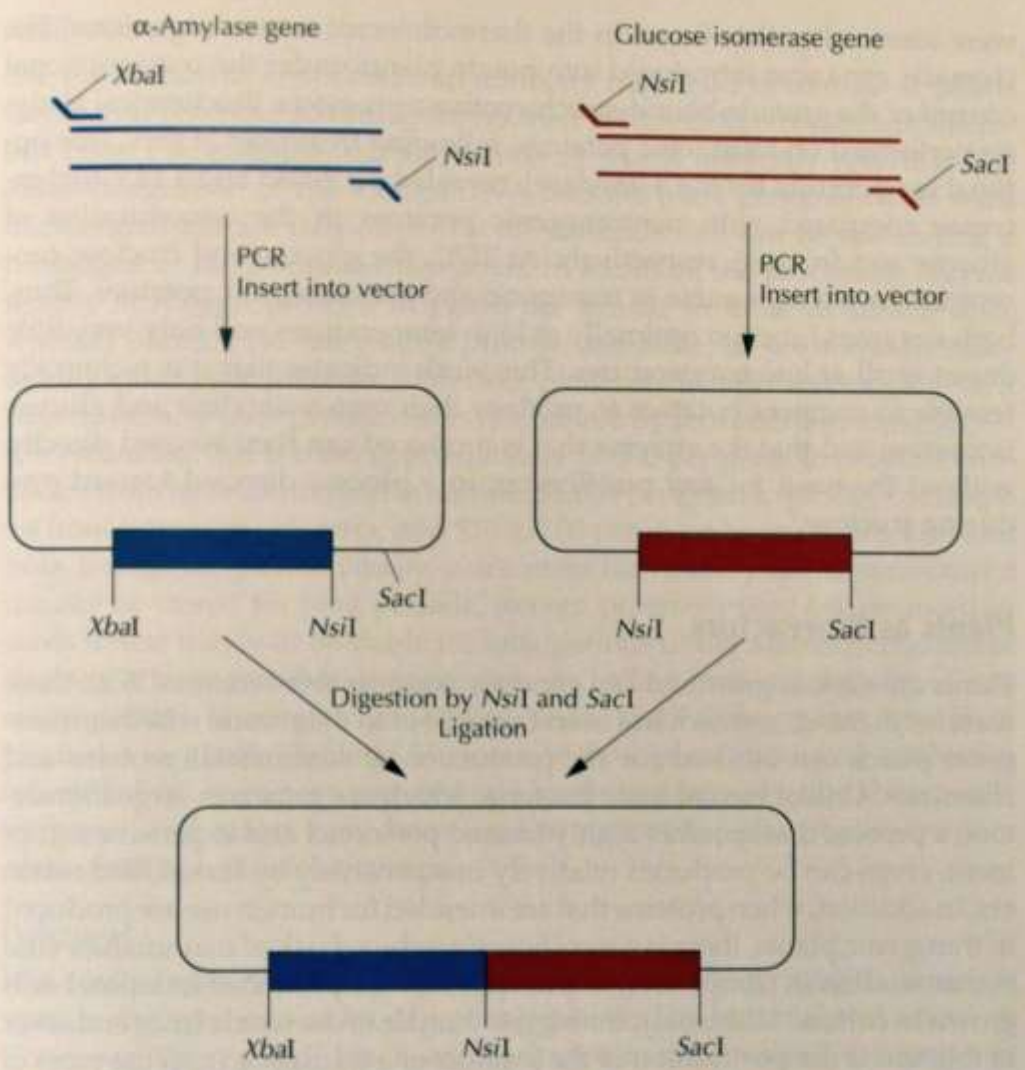


Figure 18.39 Construction of an α-amylase–glucose isomerase fusion gene. The α-amylase and glucose isomerase genes were from *Bacillus stearothermophilus* and *Thermus thermophilus*, respectively. The portions of the PCR primers that do not match the target genes are designed to retain both genes in the same reading frame and to include appropriate restriction endonuclease sites. Adapted from Beaujean et al., *Biotechnol. Bioeng.* **70**:9–16, 2000.